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Targeting of Cancer Stem Cells and Their Microenvironment in Early-Stage Mutant K-ras Lung Cancer

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14. ABSTRACT

The overall goal of this proposal is to understand the role of the Hh pathway in the maintenance and proliferation of lung adenocarcinoma progenitor cells and to establish therapeutic strategies that target the stem cell and its microenvironment for early stage lung cancer. Toward this end, we successfully labeled AlexaFluor 647 to anti-SHH/IHH antibody 5E1 and successfully FACS-sorted SHH+ and SHH- cells from high SHH-expressing lung cancer cell lines. The SHH+ cell population had increased SHH mRNA expression versus SHH- cell population although SHH- cells also expressed some increased SHH expression compared to normal HBEC7kt lung epithelia. Surprisingly, in liquid colony formation and tissue cultured proliferation assays, SHH- cells formed more colonies and proliferated faster than SHH+ cells. Further refinement of potential lung cancer stem cells will be performed using a combination of SHH/ALDH/NOTCH3 as potential stem cell markers. We will also test the hypothesis that SHH+ cell population may be a quiescent stem cell population and SHH- cells are transient amplifying cells. We have also established an orthotopic xenograft metastatic lung cancer model with HCC515 cells that express high levels of SHH. We will use this model for future studies for the prevention of cancer metastasis.

15. SUBJECT TERMS

Non-small cell lung cancer, cancer stem cells, Hedgehog pathway, metastasis, tumor epithelial-stromal interactions

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INTRODUCTION:

Lung cancer accounts for the greatest number of cancer deaths in the U.S. and the world. Five-year survival rates are <50% and ~30% for patients with stage I and II non-small cell lung cancer, respectively, despite surgery and adjuvant chemotherapy, in large part due to recurrence at the original site of disease and metastasis to distant sites. Cancer stem cells have been proposed to be the cancer cells that metastasize to other areas of the body and are resistant to chemotherapies. We have found that in mutant K-ras lung cancers, a group that comprises ~30% of NSCLC with no available targeted agents, only a subset of tumor cells have mutant K-ras activity. These cells also express high levels of secreted SHH ligand implying that mutant K-ras may be active in a cancer stem cell compartment and, analogous to lung development, use paracrine stromal Hh pathway activity to promote its growth and metastases. The *overall goal* of this proposal is to understand the role of the Hh pathway in the maintenance and proliferation of mutant K-ras lung cancer progenitor cells and to establish therapeutic strategies that target the stem cell and its microenvironment for early stage lung cancer. Therapies that target cancer stem cells and its supportive environment may prevent recurrences after treatment and possibly lead to a cure for early stage lung cancer.

KEYWORDS:

Non-small cell lung cancer
cancer stem cells
Hedgehog pathway
metastasis
tumor epithelial-stromal interactions

ACCOMPLISHMENTS:

I. Major Goals of the Project:

AIM 1. To determine that cells expressing SHH are the CSCs and that stromal Hh pathway is critical for tumor growth in mutant K-ras lung cancer. Months 1-12

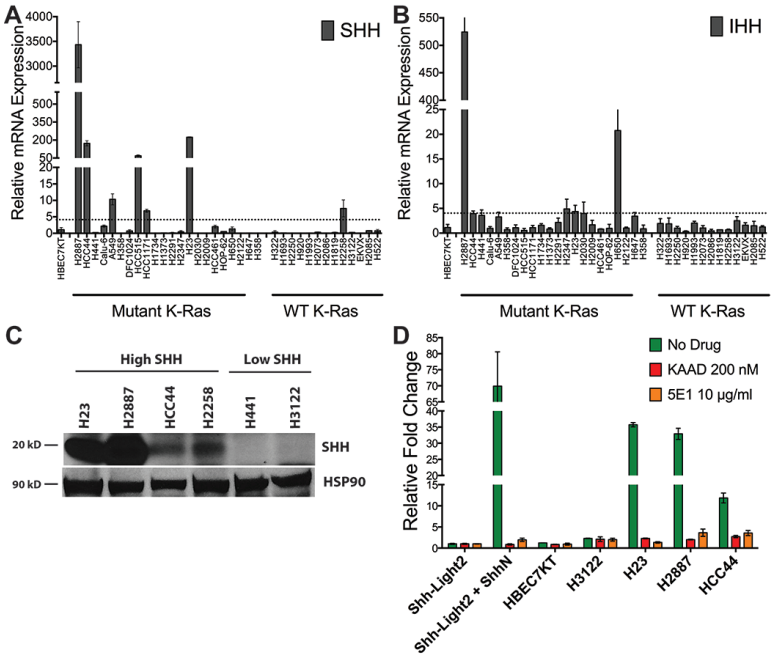

Figure 1. Human Lung Adenocarcinoma Cell Lines. (A-C) Expression of *SHH* (A) and *IHH* (B) mRNA as measured by qPCR relative to a normal bronchial epithelial (HBEC7KT) cell line. Dashed line represents 4x relative expression of HBEC7kt. (C) Immunoblot of N-terminal SHH of high and low SHH-expressing lung cancer cell lines from panel A. (D) Co-culture of high SHH-expressing H23, H2887 and HCC44 and low SHH-expressing H3122 cancer cell and HBEC7kt with Shh-Light2 reporter cell line activate the Hh pathway, which is inhibited by KAAD-cyclopamine (SMO antagonist) and 5E1 anti-SHH/IHH monoclonal antibody.

Goal 1. Isolation and testing of SHH-expressing lung cancer cells for stem cell capacity

Goal 2. Isolation of ALDH$^+$ cells and testing for SHH-expression and stem cell capacity

Goal 3. Test of ALDH$^+$SHH+ cells for stem cell capacity

AIM 2. To test drug combinations that target pathways active in tumor epithelium and the Hh pathway in stroma. Months 13-15.

Goal 1. Combination drug therapy targeting tumor epithelial and stromal Hh pathway active cells in vitro.

Goal 2. Combination drug therapy targeting tumor epithelial and stromal Hh pathway to inhibit metastases of lung cancer stem cells in vivo.

II. Accomplishments under Goals:

For Aim 1, we have increased the number of cell lines tested for *SHH* and *IHH* mRNA expression by real time PCR (qPCR) (Fig. 1 A, B) from our original proposal and have found several more cell lines that have increased expression of these Hh ligands. H23 mutant lung adenocarcinoma cell line show increased SHH mRNA expression ~200x that of the normal epithelial HBEC7kt cell line. Immunoblot analysis (Fig. 1C) reveals high SHH protein levels in H23, H2887, HCC44, and HC2258 cell lines in contrast to low SHH expression in H441 and H3122 cell in accord with our qPCR data. We have also examined H23 and H3122 cell in co-culture hedgehog (Hh) pathway signaling assay in addition to previously shown H2887 and HCC44 cell lines. Cancer cell lines are co-cultured with SHH-Light2 cells (1), a mouse fibroblast cell line with a GLI-binding site luciferase reporter. H23 cells induce high levels of Hh pathway activation in SHH-light2 cells whereas low-SHH-expressing H3122 cells do not (Fig. 1D). Furthermore, pathway activation of SHH-light2 cells co-cultured with H23 cells was abrogated by both KAAD-cyclopamine 200 nM (a potent SMO inhibitor) and the anti-SHH/IHH 5E1 (2, 3) monoclonal antibody. The inhibitory activity of 5E1 suggests that the cancer cells secrete SHH and activate the Hh pathway in Shh-Light2 fibroblasts in a paracrine manner.

We then tested whether SHH+ lung cancer cells may have cancer stem- or progenitor-like properties. To FACS-sort SHH+ cells, we utilized the anti-SHH/IHH 5E1 antibody (Fig. 1D) to directly bind the

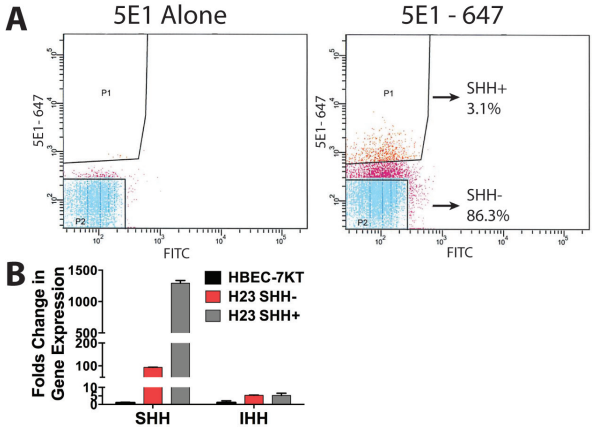

Figure 2. Isolation of SHH+ Cells from H23 lung cancer cells. (A) H23 lung cancer cells were isolated by FACS-sorting using 5E1 antibody conjugated to AlexaFluor 647 (5E1-647). Gating of positive cell lines were based on H23 cells with 5E1 (no dye; left panel). (B) qPCR of *SHH* and *IHH* mRNA of FACS-sorted cells from (A). Data are normalized to low SHH/IHH expressing normal lung epithelial cell line, HBEC7kt.

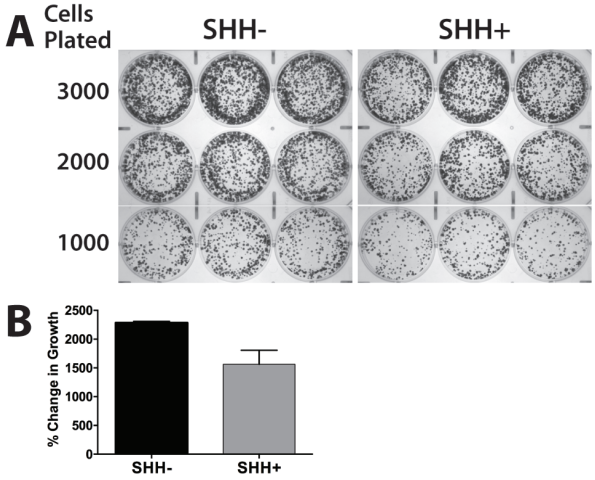

Figure 3. Growth of SHH+ and SHH- H23 cells. (A) Limiting dilution liquid colony formation assay of H23 FACS-sorted SHH- and SHH+ cells from Fig. 2A were plated at the noted number of cells per well in 6 well plates and incubated for 2 weeks. (B) Cell proliferation assay of H23 cells incubated for 8 days and cell growth monitored by Cell Titer Glo. Analogous to liquid colony formation assay, SHH- cells show greater proliferation than SHH+ cells.

Hh ligands. We labeled 5E1 antibody with AlexaFluor-647 dye (5E1-647 hereafter) using Zenon labeling kit (Thermofisher). H23 (Fig. 2A), HCC4 (Fig. 4A), and H2887 (Fig.5A) were FACS-sorted (UTSW Flow Cytometry Core Facility). Incubation of cells with 5E1-647 was performed on ice to limit the secretion of Hh ligands from the cells. Gates for high and low SHH-expression were set with cells incubated 5E1 antibodies alone (no fluorescent dye) (left panel of Fig. 2A, 4A, and 5A). Percent of cells sorted for each gate are shown within the figure panels (right panel of Fig. 2A, 4A, 5A). qPCR of the sorted cells were performed to verify that the 5E1 antibody was capable of sorting for SHH+ cells. Indeed, SHH+ H23 (Fig 2B) and H2887 (Fig. 5B) cells showed increased levels SHH mRNA expression compared to SHH- cells. However, the SHH- cell populations of both cell lines showed substantial elevations of SHH mRNA expression compared to control HBEC7kt cells. The increased SHH mRNA expression in the cells sorted as SHH- may be due to the loss of some antibody from secretion of the Hh ligands from the cells. As the majority of the cells were sorted as SHH-, we will lower the threshold of the SHH- gate in order to minimize the number of true SHH+ cells sorted in SHH- gate. FACS-sorted HCC44 cells were not subject to qPCR analysis as there were not enough viable cells from the FACS-sort. We believe this may have been due to the high speed with which the sorting occurred and can be corrected with a slower flow rate.

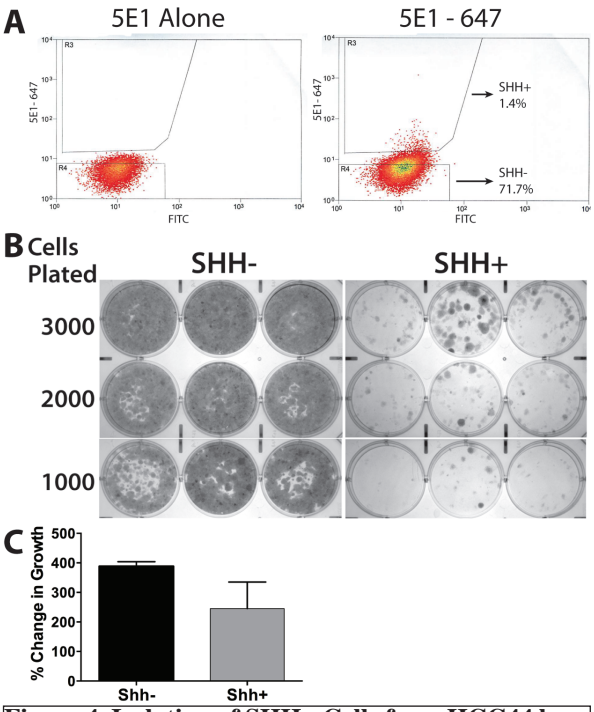

Figure 4. Isolation of SHH+ Cells from HCC44 lung cancer cells. (A) HCC44 lung cancer cells were isolated by FACS-sorting using 5E1-647 analogous to Fig. 1A. (B) Liquid colony formation assay of HCC44 FACS-sorted SHH- and SHH+ cells from Fig. 4A analogous to Fig. 3A for 2 weeks. (C) Cell proliferation assay of HCC44 cells incubated for 8 days and cell growth monitored by Cell Titer Glo. Analogous to liquid colony formation assay, SHH- cells show greater proliferation than SHH+ cells.

Proliferation and limiting dilution liquid colony formation assays were performed with SHH+ and SHH- H23 (Fig. 3) and HCC44 (Fig. 4B,C) FACS-sorted cells. For limiting dilution assays, cell numbers from 1000-3000 cells per well in a 6 well plate were laid down and then allowed to incubate for 2 weeks to form colonies. Surprisingly, for both H23 (Fig. 3A) and HCC44 (Fig. 4B), sorted SHH- cells produced more colonies than the SHH- cells. For cell proliferation assays, 5000 cells/well were plated in 24 well plates and incubated for 8 days prior to cell number measurement by Cell Titer Glo (Promega). Similar to the liquid colony formation assays, SHH- cells proliferated more quickly than SHH+ cells of H23 (Fig. 3B) and HCC44 (Fig. 4C)

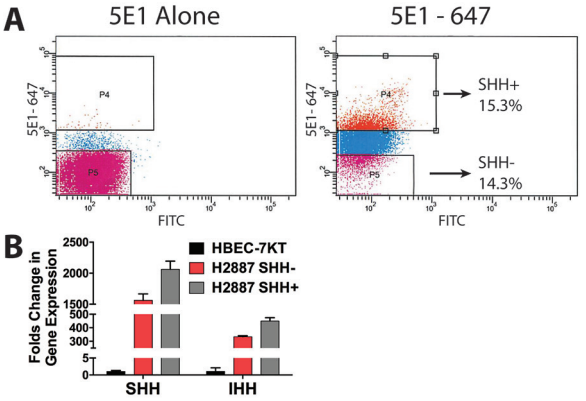

Figure 5. Isolation of SHH+ Cells from H2887 lung cancer cells. (A) H2887 lung cancer cells were isolated by FACS-sorting using 5E1-647 analogous to Fig. 2A. (B) qPCR of *SHH* and *IHH* mRNA of FACS-sorted cells from (A). Data are normalized to low SHH/IHH expressing normal lung epithelial cell line, HBEC7kt.

cells.

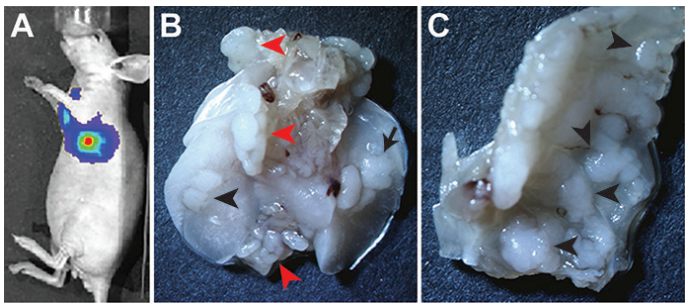

Figure 6. Orthotopic xenograft of HCC515 lung cancer cell line. One million HCC44-FL cells were injected orthotopically into left lung of nude mice. (A) Bioluminescence (BLI) imaging of mice were performed 3 weeks after injection. (B) Examination of lungs showed the primary tumor (black arrow) and metastases to mediastinal lymph nodes (red arrowheads), right lung (black arrowhead) and right chest wall (C, black arrowheads).

Part of our objectives for Aim 1 was to perform subcutaneous limiting dilution assays of SHH+ and SHH- cells in mice to test the stem cell capacity of SHH+ cells. As a pilot trial, we injected 2 million unsorted H2887, HCC44, and HCC515 high SHH-expressing cancer cells subcutaneously into flanks of 3 nude mice each. HCC515 cells formed detectable tumors within 2-3 weeks. H2887 cells took >6 weeks to form a detectable tumor whereas HCC44 cells did not form any detectable tumors after 6 months. One possibility may be that the immune system of nude mice (which lack T cells) may be competent enough to reject most of H2887 and all of HCC44 cells. We will perform this trial experiment once more except use NOD-SCID-gamma (NSG; Jackson Laboratory) mice, a more severely immunocompromised mouse strain that lack mature T cells, B cells, NK cells, and cytokine signaling to due loss of the IL2 receptor.

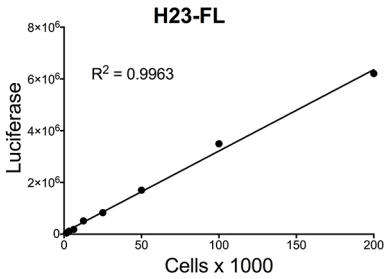

Figure 7. H23-FL lung cancer cell line. H23 lung adenocarcinoma cell line stably expressing firefly luciferase and GFP (not shown). Firefly luciferase is expressed in a linear manner with increasing cell numbers that range over 2 orders of magnitude

As part of another project, we have established an orthotopic xenograft model of lung cancer (approved by UTSW IACUC) that has relevance to his project in regard to Aim 2, Goal 2, the metastatic potential of cancer stem cells. Thus, we present the data here. One million high SHH-expressing HCC515-FL lung cancer cells stably expressing firefly luciferase (25% matrigel/PBS suspension) were injected directly into the left lung through the intercostal muscles after a small incision of the skin and visualization of the left lung. Three weeks after injection, tumors were visualized by bioluminescent imaging (BLI) (UTSW Small Animal Imaging Core Facility). Surprisingly, we saw bioluminescence in both left and right chests (Fig. 6A) when only the left lung had been injected with tumor cells. Dissection of the lungs revealed the primary tumor formed in the left lung (Fig. 6B, black arrow) and gross metastases to the mediastinal lymph nodes (Fig. 6B, red arrowheads), contralateral right lung (Fig. 6B, black arrowheads), and right chest wall (Fig 6C, black arrowheads). We believe that this metastatic lung cancer model is a more accurate model of metastasis than the tail vein injections of tumor cells we proposed in our original grant application. Toward this end, we have also established another high SHH-expressing cell line, H23-FL that stably expresses firefly luciferase and GFP (Fig. 7). We will test cell line for the ability to establish orthotopic tumors and generate metastases as with the HCC515-FL cell line (Fig. 6).

III. Opportunities for training and professional development this project has provided

1. Formal and informal one-on-one meetings with mentors Dr. John Minna and Dr. Joan Schiller

2. Faculty mentor meetings with Faculty Mentor Committee consisting of Dr. John Minna

(Professor), Dr. Michael White (Professor), and Dr. Rolf Brekken (Professor) for guidance on all aspects of science and career development.

3. Monthly UTSW-MD Anderson Lung Cancer SPORE video conference meeting.

4. Bi-weekly UTSW Lung Cancer Disease-Oriented Team meetings.

IV. How were results disseminated to communities of interest?

The results of our studies are still preliminary and have not been disseminated.

V. What do you plan to do during the next reporting period to accomplish the goals?

During the next reporting period we will address several of the concerns and issues that were brought up during this and continue with other goals and aims of the proposal.

(1) As the qPCR of SHH- cells still showed significant SHH mRNA expression, we will repeat the FACS-sort using lower threshold of 5E1-647 fluorescence levels in order to omit cells that may still have lost some SHH from the cell surface due to secretion of the protein.

(2) We will also proceed with co-staining the cells for ALDH+ and SHH+ cells with Aldefluor assay and 5E1-647 antibody. The Aldefluor assay fluoresces in the green color spectrum whereas 5E1-647 fluoresces in the far-red color spectrum. We will determine by FACS-sorting whether ALDH and SHH are expressed within the same cell population. We will also test NOTCH3 in combination with 5E1-647 alone or in combination with 5E1-647 and Aldefluor assays. Anti-NOTCH3 antibody will be labeled with Alexa Fluor 594 (red color spectrum) in an analogous manner to 5E1-594. As ALDH+ and NOTCH3+ have been reported to be markers of lung cancer stem cells (4), FACS-sorting of ALDH+/NOTCH3+/SHH+ may be able to determine a more refined population of cancer stem cells. One possible explanation for increased colony and proliferation of SHH- cells compared to SHH+ cells may be that SHH+ cells may represent a more quiescent population whereas SHH- cells may contain a transient amplifying cell population. If this is the case, then one would expect the results that we have seen. However, according to the cancer stem cell theory(5), transient amplifying cells have limited capacity for cell division whereas cancer stem cells do not. As a first pass, we will perform tumor sphere assays (6) – serum free assays enriched with EGF and FGF that enrich for stem/progenitor cells. The number of tumor spheres will be counted for ALD+/NOTCH3+/SHH+ vs ALD+/NOTCH3+/SHH- cells, spheres will be dissociated into single cell suspensions with collagenase and a second round of FACS-sorting and tumor sphere assays will be performed. This procedure will be repeated at least 5 times. If indeed the SHH+ cell population harbors a quiescent stem cell population and the SHH- stem cell population harbors a transient amplifying cell population, we anticipate that the SHH- cells will decrease the number of tumor spheres formed over successive generations whereas the SHH+ cell population should maintain the number of tumor spheres.

(3) As we now have both H23-FL and HCC515-FL high SHH expressing cell line, we will test the H23-FL cell line to generate subcutaneous, orthotopic tumors, and metastases from orthotopic tumors in NSG mice.

(4) For Aim 2, Goal 2, the metastasis model of injecting tumor cells in tail vein will be replaced by our orthotopic lung cancer model with HCC515-FL cells (Fig. 6) and possibly H23-FL cells. Ten thousand of SHH+ or SHH- HCC515-FL or H23-FL cells will be injected orthotopically in the left lung in 25% matrigel/PBS suspension. BLI images will be taken weekly until tumor formation has been detected in the left lung. Once BLI has confirmed the establishment of tumor, the experiments outlined in Aim 2.B.1 and 2.B.2 of the original proposal will be followed (IACUC

approval pending).

(5) Testing of drugs against tumor epithelia and stromal Hh pathway activity *in vitro* (Aim 2, Goal 1).

(6) Continue with breeding of *LSL-Kras$^{G12D/+}$;Trp53$^{fl/fl}$;R26R$^{luc/+}$* mouse strain.

IMPACT:

I. Impact on development of the principle discipline of the project: Nothing to report. The data are preliminary and further experiments are required for firm conclusions.

II. Impact on other disciplines: Nothing to report. The data are preliminary and further experiments are required for firm conclusions.

III. Impact on technology transfer: Nothing to report

IV. Impact of society beyond science and technology: Nothing to report. The data are preliminary and further experiments are required for firm conclusions.

CHANGES/PROBLEMS:

I. Changes in approach and reasons for change.

No significant changes were made during this reporting period. However, we did change the agent that was used to identify SHH+ cancer cells. We have generated and used a fluorescently labeled anti-SHH/IHH 5E1 monoclonal antibody that has been well validated to bind SHH/IHH in the literature and in our own studies (Fig. 1D). In our original grant application, we proposed to use commercially available fluorescent reporter promoters of SHH, package them in lentiviruses and infect them into lung cancer cell lines. Upon further reflection, this approach had some potential drawbacks including the possibility that the available promoters may not be accurate, transcription factors that induce SHH expression may bind to promoters and enhancers beyond the region represented in the commercially available product including introns of the gene. Thus this approach may not have enough sensitivity to capture most of the high SHH-expressing cells. Thus, we opted to test 5E1 labeled with AlexaFluor 647 to sort for SHH+ cells. If we are unable to further refine and discriminate between SHH+ and SHH- cells by 5E1-647, then we will attempt to use the commercially available SHH promoter with fluorescent reporter

II. Actual or anticipated problems or delays and actions or plans to resolve them.

Although the award period started on Sept. 15, 2014, mouse studies were initiated in November 2014 when the studies were approved by ACURO.

III. Changes that had a significant impact on expenditures.

None.

IV. Significant changes in use or care of human subjects, vertebrate animals, biohazards, and select agents.

No significant changes were made during this reporting period. However, for this coming research period, we will make a change in our use of mice where in Aim 2, we will substitute the orthotopic metastatic xenograft model (Fig. 6) for the injection of tumor cells into the

tail vein for our metastatic tumor models. Please refer to "Accomplishments, Section V.4" for details.

PRODUCTS:

We have produced a high SHH-expressing lung adenocarcinoma cell line, H23-FL, that stably expresses firefly luciferase and GFP under EF1a promoter. This cell line, in addition to previously made "-FL" cell lines, may be useful for both tissue cultured and *in vivo* assays to study the cancer biology of high SHH-expressing lung cancer cells.

PARTICIPANTS AND OTHER COLLABORATING ORGANIZATIONS:

I. Participant

Name:	James Kim
Project Role:	Principal Investigator
Nearest Person Month Worked	6.0
Contribution to Project:	Planned, guided and execution of experiments of this proposal

Name:	John Minna
Project Role:	Other Significant Contributor
Nearest Person Month Worked	0.0
Contribution to Project:	Mentor and provision of cell lines and other reagents.

Name:	Lawrence Lum
Project Role:	Other Significant Contributor
Nearest Person Month Worked	0.0
Contribution to Project:	Lend expertise and reagents in cell biological techniques and Wnt pathway.

Name:	Baozhi Chen
Project Role:	Research Scientist
Nearest Person Month Worked	10.8
Contribution to Project:	Execution of cell biology and mouse experiments

Name:	Alexandra Wilson
Project Role:	Research Technician II
Nearest Person Month Worked	12.0
Contribution to Project:	Breeding and genotyping of mice, support and execution of mouse and cell culture experiments

II.Change in active support for PI.

New grants since submission of grant proposal.

1. SKF-14-057 (PI: James Kim) 7/01/2014 – 06/30/2015 2.4 cal months

The Sidney Kimmel Foundation for Cancer Research

"The Interaction Of Stromal Hedgehog Pathway With Cancer Stem Cells Of Lung Adenocarcinoma"

The major goal is to characterize and develop antibody that specifically target stromal Hh pathway in lung adenocarcinoma but not in immune cells and develop therapeutic strategies with this antibody to target lung cancer stem cells.

Role: PI

2. W81XWH-14-1-0540 (PI: David Gerber) 09/01/2014 – 08/31/2017 0.6 cal months
Dept. of Defense
"Phase 0 trial of itraconazole for early-stage non-small cell lung cancer"
The major goal is to understand the biology of itraconazole as an angiogoenesis and Hh pathway inhibitor in early stage human non-small cell lung cancer.
Role: Co-investigator

3. P50-CA70907 (PI: John Minna) 7/1/2014-6/30/2019 0.0 cal months
National Institutes of Health
Project 4: "Therapeutic Targeting of Telomerase in Lung Cancer Stem Cells"
The goal of project 4 is to target telomerase in cancer stem cells of non-small cell lung cancer with IIWR-1, a Wnt and tankyrase inhibitor, and 6-thio-2'deoxyguanosine, a nucleoside-based telomerase substrate in preclinical models and a clinical trial, respectively.
Role: Collaborator

No overlap in any listed proposals with the current Dept. of Defense proposal.

III. Other Organization/Partners? Nothing to report.

SPECIAL REPORTING REQUIREMENTS: Not applicable

APPENDIX: References listed in the Annual Report

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